REMARKS

The Examiner has identified that the oath/declaration as originally submitted is defective. A new declaration and power of attorney is submitted in which the application is identified by number and by filing date. The Examiner has also requested new drawings in compliance with 37 C.F.R. 1.121(d). Replacement drawings are hereby submitted. Claims 1-20 and 47 are presently being examined. Claims 1, 2, 12, 13 and 47 have been amended and claims 3, 4 and 11 are cancelled. The subject matter of claim 11 has replaced the subject matter of claim 1 with the previous limitation of a divalent transition metal cation and RNaseIII is further limited to prokaryotic RNaseIII. Support for this amendment can be found on page 26, line 14. In claim 13, support for "purified" is provided for example in Figure 1F (page 13, line 20), pg. 47, pg. 69, line 26, and pg. 74, line 21.

The Examiner has rejected the claims under 35 U.S.C. §102(a) citing Zamore et al., and under 35 U.S.C. §102(e) citing Beach et al. (evidenced by Ketting et al.) and Yang et al. and under 35 U.S.C. §103 citing Yang in view of Gross et al.

Applicants believe it would be helpful to briefly explain the present claimed invention in the context of the limitations of the prior art. In August 2002, it was known that eukaryotic cells contained specific enzymes that digested double-stranded RNA (dsRNA) into fragments of a size suitable for silencing genes. This was demonstrated in a variety of eukaryotic cells. Researchers such as Zamore et al. examined the fate of dsRNA that was introduced into *Drosophila* cell lysates and tracked the results using radioactive

markers. Beach et al. studied the mechanism of Dicer action in cultured eukaryotic cells and in cell lysates and Ketting also studied the mechanism of Dicer from *C. elegans in vivo* and in cell lysates. The contents of the cell lysates and cells are complex so that the reaction conditions for creating the siRNA were largely unspecified. Neither Zamore et al. or Beach et al. described the purification of siRNA fragments after *in vitro* cleavage. Indeed Yang explains why this might be so on page 2, in ¶014 which states "Although Dicer is involved in the ds RNA cleavage *in vivo*, using Dicer to prepare siRNA *in vitro* may be problematic because ds RNA cleavage by Dicer is very inefficient particularly for short ds RNAs."

In contrast to Zamore et al. and Beach et al., Yang et al. used *E. coli* RNaseIII to generate RNA fragments of a size suited for gene silencing in eukaryotic cells. Unfortunately, *E. coli* RNaseIII causes double-stranded RNA to be cleaved into fragments that are too short to trigger an RNAi response. By reducing the time and temperature of incubation of enzyme with substrate, Yang reduced the production of small fragments but increased the amount of large fragments too large to trigger an RNAi response. This can be seen by the smear in Figure 1. Hence, Yang et al. failed to achieve "completion of digestion" as defined in the present application on page 27, line 1 and therefore was obliged to add time-consuming size fractionation steps to obtain a dsRNA preparation suitable for siRNA (see page 8, ¶0079). Moreover, Yang et al. did not appear to consider the teachings of Gross that the cleavage activity of RNAseIII could be altered in the presence of manganese. This is understandable because Gross et al. showed that cleavage activity increased rather than was diminished by the presence of manganese, which was opposite to the effect sought by Yang et al.

Applicants recognized the significant problems raised by the use of RNaseIII and a buffer containing magnesium and sought to improve the method of generating siRNA fragments. It was discovered by applicants quite fortuitously and contrary to the teaching of the prior art (page 21 of the above application), that the presence of divalent transition metal cations in the RNAse reaction mixture *in vitro* had a beneficial effect on generating dsRNA fragments of desired size from large dsRNA (observed as a sharp band in for example Figure 1) corresponding to siRNA. Applicants completed an extensive analysis of the conditions for optimizing the reaction as reported in the above application. A number of significant advantages for this methodology were revealed (see for example page 22 of the application). For example, it was possible to cleave substantially all large dsRNA and to generate RNA fragments less than about 50 bases without degrading the fragments. Consequently, size fractionation was not required and mixtures of fragments for siRNA could be obtained simply and efficiently by ethanol precipitation only (see page 74, line 23). It was also found that the siRNA fragments obtained by the claimed method could be mapped to the substrate dsRNA so that a substantial portion (see page 28) of the substrate sequence was represented (see for example, Figures 4A and 4B of the above application).

Zamore Reference

The Zamore reference does not anticipate amended claim 1 and dependent claims 2-10, independent claim 12, or independent claim 13 and dependent claims 14-20 in the above application, for reasons that include the following:

* Dicer is not a prokaryotic RNase III and is therefore outside the scope of the amended claims 1-12;

* In Zamore et al., dsRNA was added to a cell lysate and was cleaved in the lysate. No purification of dsRNA fragments was suggested or taught. Amended claim 13 is directed to purified RNA fragments of a size of 15-30 nucleotides.

In conclusion, the Examiner is respectfully requested to reverse the rejection.

Beach et al.

The Beach et al. reference does not anticipate amended claim 1 and dependent claims 2-10, independent claim 12, or independent claim 13 and dependent claims 14-20 in the above application, for reasons that include the following:

* Beach et al. do not utilize a prokaryotic RNaseIII and is therefore is outside the scope of the amended claims 1-12;

* In Beach et al., purification of dsRNA fragments was not suggested or taught. Amended claim 13 is directed to purified hsiRNA fragments.

Yang et al.

The Examiner has asserted on page 6 of the Office Action mailed April 4, 2006 that Yang et al. teach that

> the hsiRNAs mixture represents is complete in less than 6 hours and represents a set of overlapping fragments wherein at least one fragment or 50 to 100% are capable to cleave the target mRNA when introduced into an eukaryotic cell (p1, paragraph

> 004, p2, paragraph 0015, p6, paragraph 0045, p6 paragraph 0053 Figure 1B) (emphases added).

Applicants respectfully submit that there is no support in Yang et al. for "the hsiRNA is complete in less than 6 hours".

In fact, Yang et al. state that

> Exhaustive cleavage of dsRNA by *E. coli* RNaseIII leads to duplex products averaging 12-15bp in length. These short dsRNA are unable to trigger an RNAi response in mammalian cells (page 2, ¶0015)

According to the above, exhaustive cleavage results in fragments of dsRNA that are too small for RNAi. Certainly 6 hours or 4 hours or 2 hours or indeed a few minutes of digestion with RNaseIII in the presence of magnesium ions could significantly degrade dsRNA to a size that is unsuited for siRNA. There is therefore no basis for the Examiner's assumption that the Yang reference describes or is enabled for the production of hsiRNA (greater than about 15% of the total number of fragments would have a length of 18-25 base pairs) as defined on page 24 of the application nor that digestion was completed as defined on page 27 of the application.

> We found that Gst-RNase III fusions were highly active in cleaving these dsRNAs at 37°C..... (page 6, ¶0053)

Based on the comments in ¶0015, it can be deduced that at 37°C, the highly active fusion enzyme produced RNA fragments that were too short to accomplish RNAi.

> ...After optimization we found that <u>limited</u> RNase III digestion of dsRNA at room temperature for 1 hour yielded

> ample amounts of esiRNA for inhibition of most genes. (page 6, ¶0053) (emphases added).

There is no suggestion in the above citation that RNaseIII could generate hsiRNA as defined quantitatively by the applicants on page 24 of the above application. Moreover, because RNaseIII in the presence of magnesium ions results in rapid degradation of large dsRNA, it is likely that at 37°C most material was degraded. It is also possible that at room temperature, most material was undigested or degraded. It is likely that large amounts of starting material were required to produce "ample amounts of esiRNA for inhibition of most genes". There is no teaching by Yang et al. as to what "most genes" refers to. Clearly, RNAi appeared to work sometimes but the variability was not explained.

The figures provided in the Yang reference do not assist in interpreting the above. Indeed Figure 1B, which refers to 4% agarose gels (a poor discriminator of sizes for fragments less than 200 base pairs), shows a gradient of bands of different sizes at 15-minute incubations. These results reflect not only the poor resolution of agarose gels for this size range of polynucleotide but also the difficulty of firstly: providing enough RNaseIII to achieve any cleavage, and secondly, preventing total cleavage of dsRNA into 12-15bp fragments.

Applicants have attached a page from Sambrook et al. "Molecular Cloning-A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press (1989) page 6.2 that explains that agarose gels are suitable for separating fragments of 200bp - 50kb and not smaller fragments for example less than 50bp in size.

The Examiner further states that "The ratio of RNaseIII to dsRNA is in the range of about 0.0125:1 (page 8, ¶0079)" (emphasis added).

Applicants respectfully submit that page 8, ¶0079 describes an enzyme to substrate ratio of .01:1 and .005:1 only. No range of ratios is provided by Yang et al. Yang et al. is required to use a low ratio of enzyme to substrate to limit digestion and minimize the rapid degradation of dsRNA by RNaseIII in the presence of magnesium ions that is otherwise observed.

In contrast to Yang et al., Applicants described titrations to identify preferred ratios of enzyme to substrate under specified defined conditions (Figures 1C and 1D). A much higher ratio of enzyme to substrate (greater than 0.25:1) was found by Applicants to be preferable compared with the ratios used by Yang et al. (0.005:1 and 0.01:1).

The Examiner states that "Yang et al. teach reaction conditions that efficiently generate 20-25 bp siRNAs (ie more than 30% (see P2, paragraph 0015)". The Examiner has deduced that the term "efficient" encompasses a value of more than 30% in reference to the present dependent claim 16. "Efficiency" is a relative term as used by Yang et al. Exhaustive cleavage results in products that average 12-15bp. In these digestions there is no detectable dsRNA on a gel of a size suited for RNAi. In contrast, when Yang et al. did a limited digestion (Figure 1), a smear was obtained that apparently includes an undetermined amount of fragments having a size suited for siRNA. This is referred to by Yang et al. as efficient generation of 20-25bp fragments. It may refer to 0.1% or 1% of the total number of fragments.

With respect to claims 14 and 15, Yang et al. make no reference to the portion of a sequence represented by RNaseIII cleavage products illustrated in Figures 4B and 4C.

There is no suggestion or teaching in Yang et al. that a divalent transition metal cation be used in addition or instead of the standard Mg^{++} in the RNaseIII reaction mixture. Yang et al. do not suggest or teach the benefit of using a divalent transition metal cation as a cofactor for RNAseIII activity to generate hsiRNA fragments although Yang et al. do acknowledge that *E. coli* RNase III leads to duplex products averaging 12-15bp in length, which cannot trigger an RNAi response (page 2, ¶0015).

Yang et al. do not anticipate the claimed invention for reasons that include the following:

* Yang et al. do not teach the ratio of enzyme to substrate required in amended claims 1-12. Yang et al. utilize a ratio of enzyme: substrate of .01 or .005: 1 compared with the required ratio of "greater than or equal to .25: 1" required in claims 1-12 of the present claimed invention.

* Yang et al. teach the use of magnesium ions and not divalent transition metal cations in the reaction mixture to produce dsRNA fragments.

* Yang et al. do not describe and are not enabled for a set of fragments described in claim 13 wherein "... the fragments in the set collectively represent a substantial portion of a sequence of one or more large double stranded RNAs from which the fragments are derived by *in vitro* cleavage with a purified enzyme ...". Even if Yang et al. would have considered this analysis, excessive cleavage of

template dsRNA on one hand and "limited" digestion on the other would be expected to result in substantially incomplete representation of the template sequence in fragments of size 15-30 nucleotides. The observed gene silencing for "most genes" does not speak to the extent of sequence representation of a large dsRNA by the RNaseIII/Magnesium cleaved fragments.

Yang et al. in view of Gross et al.

Applicants respectfully assert that the Examiner's rejection of the claimed invention by combining Yang et al. with Gross et al. is based on hindsight. There is no motivation by either reference to support their combination. In fact, the references teach away from each other and cannot readily be combined.

There is no suggestion by Yang et al. that a divalent transition metal cation should be added to the reaction mixture. Indeed, Gross et al. teach generating smaller fragments than otherwise obtained in the presence of magnesium ions when digesting a single strand RNA template (T7 mRNA) folded into a hairpin (see page 440 of the reference showing a denaturing gel comparing single strand RNA cleaved with RNaseIII and magnesium or manganese ions). In contrast, Yang et al. teach avoiding generating small fragments by performing limited digestions with RNaseIII and magnesium.

Additional features that differentiate the approach by Gross et al. from that by Yang et al. is the significance of salt concentration on cleavage. Gross et al. teach that stringency of cleavage is reduced at low salt concentrations. Therefore, Gross et al. teach use of low salt concentrations (less than 50mM) while Yang et al. use higher

concentrations of salt in reactions for limiting cleavage, highlighting the difference in purpose of these two references.

In summary, Yang et al. seeks to reduce RNaseIII cleavage and Gross et al. seeks to increase RNaseIII cleavage. The Examiner is respectfully requested to reverse the rejection of obviousness of the claimed invention based on the Examiner's combination of these two opposing references.

CONCLUSION

For the reasons set forth above, Applicants respectfully submit that this case is in condition for immediate allowance. Early and favorable consideration leading to prompt issuance of this Application is earnestly solicited.

Applicants petition for a three-month extension of time to file a response. A check in the amount of $510 is enclosed, covering the fees for the extension and additional claims. Please charge any deficiencies or credit any overpayment to Deposit Account No. 14-0740.

Respectfully submitted,

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Electrophoresis through agarose or polyacrylamide gels is the standard method used to separate, identify, and purify DNA fragments (electrophoresis of RNA is discussed in Chapter 7). The technique is simple, rapid to perform, and capable of resolving fragments of DNA that cannot be separated adequately by other procedures, such as density gradient centrifugation. Furthermore, the location of DNA within the gel can be determined directly by staining with low concentrations of the fluorescent intercalating dye ethidium bromide; bands containing as little as 1–10 ng of DNA can be detected by direct examination of the gel in ultraviolet light (Sharp et al. 1973). If necessary, these bands of DNA can be recovered from the gel and used for a variety of cloning purposes.

Agarose and polyacrylamide gels can be poured in a variety of shapes, sizes, and porosities and can be run in a number of different configurations. The choices within these parameters depend primarily on the sizes of the fragments being separated. Polyacrylamide gels are most effective for separating small fragments of DNA (5–500 bp). Their resolving power is extremely high, and fragments of DNA that differ in size by as little as 1 bp can be separated from one another. Although they can be run very rapidly and can accommodate comparatively large quantities of DNA, polyacrylamide gels have the disadvantage of being more difficult to prepare and handle than agarose gels. Polyacrylamide gels are run in a vertical configuration in a constant electric field.

Agarose gels have a lower resolving power than polyacrylamide gels but have a greater range of separation. DNAs from 200 bp to approximately 50 kb in length can be separated on agarose gels of various concentrations. Agarose gels are usually run in a horizontal configuration in an electric field of constant strength and direction. Larger DNAs, up to 10,000 kb in length, can be separated by pulsed-field gel electrophoresis, in which the direction of the electric flux is changed periodically. Each of these types of gel electrophoresis will be discussed in detail in this chapter.